Endothelium-derived prostacyclin: effect of serum from women with normal and hypertensive pregnancy

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1. Pregnancy-induced hypertension (or pre-eclampsia) is characterized by vasoconstriction, platelet aggregation and altered capillary permeability, implying disordered endothelial function and structure. Serum from women with pregnancy-induced hypertension has been reported by others to be cytotoxic to endothelial cells in vitro. We hypothesized that such serum contains a factor that limits the ability of endothelial cells to produce and/or release prostacyclin.

2. Prostacyclin production by intact and damaged cultured human umbilical vein endothelial cells was measured after incubating these cells with serum from non-pregnant and normal pregnant women and women with pregnancy-induced hypertension. Confluent human umbilical vein endothelial cell monolayers (intact and damaged) were incubated with sera for 24 h at 37°C followed by 1 h of incubation with added thrombin (stimulated production) or media (basal production). Supernatants were then collected for measurement of 6-keto-prostaglandin F\(_{1\alpha}\) by radioimmunoassay.

3. Basal production of 6-keto-prostaglandin F\(_{1\alpha}\) was greater in response to serum from non-pregnant women than to that from pregnant women. Within each group, sub-lethally damaged cells had a similar basal production of 6-keto-prostaglandin F\(_{1\alpha}\) to that of intact cells.

4. Basal production of 6-keto-prostaglandin F\(_{1\alpha}\) by intact or damaged cells incubated with sera from normal pregnant women and from women with pregnancy-induced hypertension was similar.

5. In all groups the addition of thrombin to intact endothelial cells increased 6-keto-prostaglandin F\(_{1\alpha}\) production approximately 15-30-fold over basal levels, but only three- to five-fold in damaged endothelial cells. Stimulated production of 6-keto-prostaglandin F\(_{1\alpha}\) by intact or damaged cells was similar with sera from normal pregnant women and women with pregnancy-induced hypertension.

6. These results indicate that any cytotoxic factor present in the sera of women with pregnancy-induced hypertension is unlikely to act by reducing basal endothelial production of prostacyclin. Secondly, if endothelial cell injury occurs in these women they may have a reduced capacity to increase endothelial production of prostacyclin in response to thrombin and possibly other important physiological stimuli.

Key words: endothelium, pregnancy, pregnancy-induced hypertension, prostacyclin, thrombin.

INTRODUCTION

Pregnancy-induced hypertension (PIH) is a common multi-system disorder with widespread manifestations involving maternal kidneys, liver and brain and the placenta [1]. It is characterized by intravascular coagulation [2], increased sensitivity to pressor agents such as angiotensin II [3] and decreased plasma volume with enhanced capillary permeability [4].

Two features central to PIH are vasoconstriction and platelet aggregation. Attention has therefore focused on a possible role for prostacyclin (PG\(_{1\alpha}\)) deficiency in the development of PIH, as PG\(_{1\alpha}\), produced primarily by vascular endothelium, is a potent vasodilator and inhibitor of platelet aggregation.

It has recently been proposed that PIH is a disorder of endothelial cell cytotoxicity [5]. Serum from women with PIH was found to be more cytotoxic to endothelial cells in vitro than serum from normal pregnant women. In the light of this evidence, we hypothesized that a factor in the serum of women with PIH might limit the production of PG\(_{1\alpha}\) by normal or damaged endothelial cells. The aim of this study was to determine whether basal and/or stimulated production of PG\(_{1\alpha}\) by cultured human umbilical vein endothelial cells (HUVEC) differed when these cells were incubated with sera from normal non-pregnant women, normal pregnant women or women with PIH.

MATERIALS AND METHODS

Subjects

Serum derived from whole blood was collected from non-pregnant women, normal pregnant women and women with PIH, and was stored at \(-20^\circ\)C. PIH was
defined as the development of blood pressure greater than 140/90 mmHg or a rise in diastolic blood pressure of more than 25 mmHg from first-trimester levels occurring in a primigravida after 20 weeks gestation with no prior history of hypertension or renal disease, whose blood pressure returned to normal within 3 months post partum. Severe PIH was diagnosed if any of the following criteria were met: blood pressure greater than 170/110 mmHg, proteinuria ≥ 2+ on dipstick testing, abnormal liver function, neurological disease or thrombocytopenia. Normal pregnant women were out-patient primigravidae attending our antenatal clinic, and non-pregnant women were healthy volunteers recruited from laboratory and nursing staff. No subject was taking any medications at the time of study. This project was approved by the Ethics Committee of the Southern Sydney Area Health Service.

Endothelial cell culture

HUVEC were chosen as they are readily available and are known to produce 6-keto-prostaglandin F₁α (6-keto-PGF₁α). Although HUVEC are of fetal origin, the hypothesis being tested was that serum induced altered cellular function rather than the endothelial cell being intrinsically abnormal. Accordingly, the origin of the cell was less important than its availability and ability to produce 6-keto-PGF₁α for the purposes of this study.

Endothelial cells were isolated from human umbilical veins by a modification of the method of Jaffe et al. [6]. Briefly, umbilical cords of normal uncomplicated pregnancies obtained within 4 h of delivery were cannulated and the vein was washed with Hartmann’s solution (containing 10 mmol/l glucose, 2.2 mmol/l Na₂HPO₄ and 0.26 mmol/l KH₂PO₄) and was incubated with collagenase, 260 units in 5 ml of Medium 199 (Gibco, Grand Island, New York, U.S.A.), for 10 min at 37°C. Five millilitres of Medium 199 supplemented with 20% (v/v) fetal bovine serum (MultiSer; Cytosystems, Castle Hill, Australia), endothelial cell growth factor (20 ng/ml), penicillin G (5 units/ml), streptomycin sulphate (5 µg/ml), heparin sodium (porcine mucous, 10 units/ml) and 20 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes; Calbiochem, La Jolla, CA, U.S.A.) were then passed through the vein and the resulting 10 ml of cell suspension was centrifuged for 5 min at 200 g. The pelleted cells were resuspended in 10 ml of supplemented Medium 199, seeded on to gelatin-coated 25 cm² flasks (Flow Laboratories, Rickmansworth, Herts, U.K.) and incubated at 37°C in a humidified atmosphere composed of 5% CO₂ and 95% air. The next day the cells were washed three times in Hanks balanced salt solution (HBSS). Fresh supplemented Medium 199 was then added and was changed every second day until the cells reached confluence (5–10 days). At confluence, endothelial cells cultured from four to five different umbilical cords (to reduce the inherent variability of PG₁ production by individual HUVEC cultures [7, 8]) were washed three times with phosphate-buffered saline (PBS), trypsinized with 0.05% trypsin and 0.02% EDTA (Flow Laboratories, Rickmansworth, Herts, U.K.), pooled and passaged on to gelatin-coated 24-well plates (2 cm²/well; Linbro Flow Lab, Virginia, U.S.A.) at a density of approximately 7 × 10⁴ cells/well. The next day each well was washed three times with HBSS and replaced with supplemented Medium 199 without heparin, as heparin has been shown to reduce the production of PG₁ by HUVEC [9]. Supplemented medium without heparin was changed every second day until the cells reached confluence (4–6 days). Only passage 1 cells were used for these experiments, and cells were identified as endothelial cells by their ‘cobblestone’ monolayer appearance and the expression of factor VIII, as detected by immunofluorescence.

Experimental protocol

On the day of the experiment the confluent HUVEC monolayers in the 24-well plates were gently washed three times with HBSS. Twelve wells of each plate were subjected to oxidant damage (1 ml of 5 mmol/l hydrogen peroxide in Medium 199 for 20 min at 37°C) as described previously [8] while the rest remained intact (1 ml of Medium 199 for 20 min at 37°C). All wells were

Table 1. Basal production of PG₁ by intact and damaged endothelial cells. Data are from the two experiments which included serum from all groups of women. Values are medians (interquartile ranges). Statistical significance: *P < 0.05, **P < 0.01 compared with serum from non-pregnant women.

<table>
<thead>
<tr>
<th>Serum from...</th>
<th>Absolute values (ng ml⁻¹ of serum mg⁻¹ of protein)</th>
<th>Corrected values (% of control serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-pregnant women (n = 10)</td>
<td>Normal pregnant women (n = 15)</td>
</tr>
<tr>
<td>Damaged cells</td>
<td>204 (156–443)</td>
<td>96* (61–293)</td>
</tr>
</tbody>
</table>
then washed gently three times with HBSS and were incubated with Medium 199/Hepes containing 20% (v/v) serum (non-pregnant, normal pregnant or PIH, equally numbered per plate), filtered with Millipore Millex-GS filter units. In preliminary experiments no difference in the production of PGI₂ by endothelial cells was found when 20% (v/v) serum was filtered with Millipore Millex-GS or Millex-GV (low protein binding) filter units. Also, significant PGI₂ production by HUVEC was not observed after incubation with serum for only 1 h. In view of this and the reported cytotoxicity after prolonged incubation [5], we incubated cells with sera for 24 h in these studies. One millilitre of each 20% (v/v) serum sample was also incubated in a well not containing HUVEC for 24 h at 37°C to determine the endogenous concentration of immunoreactive 6-keto-PGF₁₀. As controls, wells containing 1 ml of Medium 199/Hepes alone and 20% (v/v) serum containing 10 µmol/l indomethacin were included in the experimental protocol.

After 24 h, 100 µl of Medium 199/Hepes alone or thrombin (1 unit/ml, final concentration) was added to each well and allowed to incubate for a further 60 min at 37°C. Medium 199/Hepes alone (100 µl) was added for a further 60 min to the wells not containing HUVEC.

At the end of the 25 h of incubation, the serum supernatant was removed from each well, centrifuged immediately for 2 min at 12 000 g and was stored at -70°C until assayed for 6-keto-PGF₁₀. The endothelial monolayers were then washed and digested for measurement of protein content. In order to account for inherent variation in the production of PGI₂ by different batches of pooled HUVEC, a control serum was included in each tissue culture experiment and the results were subsequently corrected as described below.

### Measurement of 6-keto-PGF₁₀
6-Keto-PGF₁₀, a stable metabolite of PGI₂, was measured by radioimmunoassay by incubating 6-keto-PGF₁₀ standard (Cayman Chemical, Ann Arbor, MI, U.S.A.) or unextracted serum supernatant with ³H-labelled 6-keto-PGF₁₀ (New England Nuclear, Boston, MA, U.S.A.) and 6-keto-PGF₁₀ antiserum (Sigma) at 4°C for 18–24 h. Bound and free ligands were separated using dextran-coated charcoal and the supernatant (bound fraction) was counted by using a Packard Liquid Scintillation Counter. The limit of detection of the assay was 7 pg/100 µl, and the intra- and inter-assay coefficients of variation were 8% and 13%, respectively.

### Protein assay
Cellular protein was determined by the modification described by Oyama & Eagle [10] of the method of Lowry et al. [11]. After collection of the serum supernatant, endothelial cell monolayers were washed five times with HBSS and were digested with 400 µl of ‘Lowry solution C’ [2% (w/v) Na₂CO₃, 0.4% NaOH, 0.01% CuSO₄ and 0.027% sodium potassium tartrate] for at least 1 h at 37°C. To the cell digest in a final volume of 1 ml of ‘Lowry solution C’ plus 200 µl of distilled water was added 100 µl of Folin reagent. Cellular protein concentration was determined by using a standard curve, where 200 µl of BSA (fraction V; Sigma, Poole, Dorset, U.K.; 0–200 µg/ml of distilled water) was mixed with 1 ml of ‘Lowry solution C’ 10 min later with 100 µl of Folin reagent (1 mol/l). The absorbances of the standards and samples were read at 660 nm 30–120 min after the addition of Folin reagent.

### Data analysis
Results were collated from three separate tissue culture experiments. The serum samples from normal pregnant women and the women with PIH were equally proportioned between the three experiments but the serum samples from non-pregnant women were only included in the first two experiments. The collated results were expressed as: (i) absolute values (ng of 6-keto-PGF₁₀ ml⁻¹ of serum mg⁻¹ of protein), (ii) corrected values (percentage of control serum values) using the following formula:

\[
\text{Corrected values} = \frac{\text{Absolute values} \times 100}{\text{Control serum}}
\]

### Table 2. Basal production of PGI₂ by intact and damaged endothelial cells. Data are from all three experiments. Values are medians (interquartile ranges). Differences were not statistically significant.

<table>
<thead>
<tr>
<th>Serum from...</th>
<th>6-Keto-PGF₁₀ production</th>
<th>Absolute values</th>
<th>Corrected values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng ml⁻¹ of serum mg⁻¹ of protein)</td>
<td></td>
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</tr>
<tr>
<td>Serum from...</td>
<td>Normal pregnant women</td>
<td>Women with PIH</td>
<td>Normal pregnant women</td>
</tr>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 21)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>Intact cells</td>
<td>79 (51–180)</td>
<td>87 (66–165)</td>
<td>82 (52–98)</td>
</tr>
<tr>
<td>Damaged cells</td>
<td>94 (56–200)</td>
<td>135 (97–220)</td>
<td>64 (40–97)</td>
</tr>
</tbody>
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The collated results were expressed as: (i) absolute values (ng of 6-keto-PGF₁₀ ml⁻¹ of serum mg⁻¹ of protein), (ii) corrected values (percentage of control serum values) using the following formula:

\[
\text{Corrected values} = \frac{\text{Absolute values} \times 100}{\text{Control serum}}
\]
and (iii) stimulation index (the ratio of thrombin-stimulated to basal 6-keto-PGF$_{1\alpha}$ production for each serum supernatant).

Although 6-keto-PGF$_{1\alpha}$ was not usually detected in the 20% serum samples alone, when detectable amounts were found these values were subtracted from those obtained in the corresponding incubated samples to derive the true endothelial production of 6-keto-PGF$_{1\alpha}$. Differences between groups were analysed by using the Mann-Whitney U-test, and differences within groups by using the Wilcoxon signed-rank test. All results were expressed as medians and interquartile ranges.

RESULTS

Subjects

Serum was obtained from 10 non-pregnant women, 20 normal pregnant women and 21 women with PIH (10 mild, 11 severe). Subjects were age-matched and pregnant women were studied at a mean gestation of 35 weeks in each group. There were no differences in 6-keto-PGF$_{1\alpha}$ production between women with mild or severe disease and these results were pooled.

Basal production of PGI$_2$ by intact and damaged endothelial cells

Sera from non-pregnant women, normal pregnant women and women with PIH all stimulated intact HUVEC to produce 6-keto-PGF$_{1\alpha}$ over the 25 h incubation period. Table 1 shows that endothelial cells incubated with serum from non-pregnant women produced significantly more 6-keto-PGF$_{1\alpha}$ than those incubated with sera from normal pregnant women (P<0.05). However, there was no difference in the basal serum-induced production of 6-keto-PGF$_{1\alpha}$ between non-pregnant women and those with PIH.

Sera from all three groups also stimulated damaged endothelial cells to produce 6-keto-PGF$_{1\alpha}$ over the 25 h incubation period (Table 1). Again, endothelial cells incubated with serum from non-pregnant women produced greater amounts of 6-keto-PGF$_{1\alpha}$ than did cells incubated with sera from normal pregnant women (P<0.05).

There were no differences in the basal production of 6-keto-PGF$_{1\alpha}$ from either intact or damaged cells using serum from normal pregnant women and those with PIH (Table 2).

Stimulated production of PGI$_2$ by intact and damaged endothelial cells

Stimulation of both intact and damaged endothelial cells by thrombin significantly increased serum-induced 6-keto-PGF$_{1\alpha}$ production in all three groups (P<0.001) (Fig. 1), but the ability of damaged cells to stimulate 6-keto-PGF$_{1\alpha}$ production in response to thrombin was six- to eight-fold less than that of intact cells in each group (P<0.01) (Fig. 2).

Basal and stimulated production of 6-keto-PGF$_{1\alpha}$ by intact endothelial cells was totally inhibited by indo- methacin, and less than 20% of both intact and damaged cells were viable after 25 h when incubated with Medium 199/Hepes alone. Neither basal nor stimulated 6-keto-PGF$_{1\alpha}$ production correlated significantly with plasma uric acid concentration or blood pressure in those women with PIH.

Cellular protein

The mean cellular protein content of the intact HUVEC was 18±4 µg/well, and there were no differences in cellular protein content among the three groups studied. The mean cellular protein content of the sublethally damaged cells was 10±4 µg/well and again there were no differences in cellular protein content among the groups. The protein content of damaged cells was significantly lower than that of intact cells (P<0.001).

DISCUSSION

Sera from non-pregnant women, normal pregnant women and women with PIH all stimulated endothelial
cells to produce PGI₂. Subtle cellular damage did not alter this effect of serum upon the basal production of PGI₂ but it significantly reduced the ability of endothelial cells to stimulate PGI₂ production in response to thrombin in all three groups.

In this study we measured 6-keto-PGF₁α after 25 h of incubation with serum, as we could not demonstrate significant production after only 1 h, in keeping with the findings of Tremoli et al. [12]. As basal and stimulated 6-keto-PGF₁α production by intact cells was totally inhibited by indomethacin, the 6-keto-PGF₁α measured in the serum supernatant must have been due to stimulation by a serum component, and not due to disruption of cells and release of preformed PGI₂.

MacIntyre et al. [13] demonstrated that endothelial cell production of PGI₂ was stimulated by incubation with human plasma, and others have confirmed this in bovine [12, 14, 15], porcine [9, 16] and human [12, 17] endothelial cells. The plasma/serum component termed 'PGI₂-stimulating factor' is believed to be an important factor in the regulation of PGI₂ production by the vascular wall in vivo, and deficiency of such a factor may lead to reduced PGI₂ production and widespread microvascular thrombosis [18]. The precise nature of this factor is unknown, although it has been proposed to be uric acid [19]. This is of interest as Remuzzi et al. [20] demonstrated increased PGI₂-stimulating activity in plasma from women with PIH, who have high plasma uric acid concentrations compared with normal pregnant women. However, we found no correlation between plasma uric acid concentration and either basal or stimulated production of 6-keto-PGF₁α in those women with PIH.

An unexpected finding was that basal production of 6-keto-PGF₁α by both intact and damaged endothelial cells was significantly greater in response to serum from non-pregnant women than in response to that from normal pregnant women. This implies that serum from non-pregnant women contains greater stimulating activity or that pregnant women produce a factor which partially inhibits PGI₂ stimulation. To our knowledge this has not been described previously and needs to be confirmed using other sources of endothelial cells.

Reduced PGI₂ production has been reported in umbilical artery [21-24], uterine vessels [25] and placenta [21, 25, 26] from women with PIH. However, the total production of PGI₂ in placenta and uterine vessels is small and it is unlikely that this could produce the widespread maternal effects seen in PIH. This study shows that serum from normal pregnant women and women with PIH induced equivalent basal and stimulated endothelial 6-keto-PGF₁α production. Although subtle cell damage did not alter this basal production, it reduced the ability of endothelial cells to produce 6-keto-PGF₁α when stimulated with thrombin. Thus, if endothelial cell injury occurs in PIH and triggers platelet and thrombin activation, these cells may not be able to enhance PGI₂ production and a necessary 'defence' against further coagulation and vasoconstriction will be lost.

Table 1 suggests that serum from women with PIH produces a 6-keto-PGF₁α response intermediate between that of normal pregnant and non-pregnant women. This is similar to other changes in PIH, such as the pressor response to angiotensin II [3], reduced plasma renin concentration [27] and reduced plasma volume [4]. However, there was considerable variation in the 6-keto-PGF₁α response in this study, and very large numbers of subjects would need to be studied to validate this observation.

There are a number of caveats in relation to our findings. First, it is possible that endothelial cells require longer exposure, perhaps days, weeks or months, to a cytotoxic factor in serum before their functional abilities become impaired. Rodgers et al. [5] noted cytotoxicity only after 48 h of incubation, and it is well known that the clinical symptoms of PIH are not apparent until the later stages of pregnancy. Secondly, a cytotoxic factor may have a very short half-life, and therefore the biological activity of the factor may have been lost during the collection of blood or the storage of the serum. Thirdly, cultured HUVEC may not be the optimum cells for examining PGI₂ production. These cells require at least 16 h to be stimulated by serum [12], whereas bovine and porcine endothelial cells can be stimulated to produce PGI₂ by serum within 1 h [9, 12]. Fourthly, plasma-
derived serum, rather than whole-blood serum, may be more appropriate for measuring effects on endothelium-derived PGJ₂, as serum contains platelet-release products such as platelet-derived growth factor and arachidonic acid, both known stimulators of PGJ₂ production [28, 29]. Nevertheless, our data show that serum from non-pregnant women stimulates PGJ₂ production by HUVEC more than serum from normal pregnant women, although the reasons for this are unclear, and studies of the same women during pregnancy and post partum will be required to examine this further. Serum from women with PIH does not impair PGJ₁ production by HUVEC compared with serum from normal pregnant women.

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REFERENCES